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Grady et al., J. Biol. Chem. (1995), 270(9), 4603-11.

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Volume 270, Number 9, Issue of March 3, 1995 pp. 4603-4611

Direct Observation of Endocytosis of Gastrin Releasing Peptide and Its Receptor (*)

(Received for publication, September 12, 1994)

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ABSTRACT

Endocytosis of the gastrin releasing peptide receptor (GRP-R) may regulate cellular responses to GRP. We observed endocytosis in transfected epithelial cells by confocal microscopy using cyanine 3-GRP (cyanine 3.18-labeled gastrin releasing peptide) and GRP-R antibodies. At 4 °C, cy3-GRP and GRP-R were confined to the plasma membrane. After 5 min at 37 °C, ligand and receptor were internalized into early endosomes with fluorescein isothiocyanate-transferrin. After 10 min, cy3-GRP and GRP-R were in perinuclear vesicles, and at 60 min cy3-GRP was in large, central vesicles, while GRP-R was at the cell surface. We quantified surface GRP-R using an antibody to an extracellular epitope and an ^{125}I-labeled secondary antibody. After exposure to GRP, there was a loss and subsequent recovery of surface GRP-R. Recovery was unaffected by cycloheximide, and thus independent of new protein synthesis, but was attenuated by acidotropic agents, and therefore required endosomal acidification. Internalization of ^{125}I-GRP, assessed using an acid wash, was maximal after 10-20 min, and was clathrin-mediated since it

was inhibited by hyperosmolar sucrose and phenylarsine oxide. Thus, GRP and its receptor are rapidly internalized into early endosomes and then dissociate in an acidified compartment. GRP is probably degraded whereas the GRP-R recycles.

INTRODUCTION

Gastrin releasing peptide (GRP) ([1])is a widely distributed neuropeptide with multiple functions which is structurally related to the amphibian peptide bombesin(1) . GRP and bombesin stimulate endocrine and exocrine secretions in several tissues, induce contraction of smooth muscle, and are growth factors for normal cells and cancer cell lines(1, 2, 3, 4) . These actions are mediated by a high affinity interaction with the seven transmembrane domain, G protein-coupled GRP receptor (GRP-R)(5, 6) . Little is known about regulation of the cellular location and signaling of the GRP-R. This regulation would be expected to affect the responsiveness of cells to GRP.

The response of many cells expressing the GRP-R desensitizes and resensitizes after exposure to agonist(7, 8, 9) . The mechanism of desensitization is unknown, but may include phosphorylation of the receptor with subsequent uncoupling from G proteins and physical removal of the receptor from the plasma membrane by endocytosis(8) . Another neuropeptide, substance P, induces endocytosis and recycling of the neurokinin 1 receptor which coincides with desensitization and resensitization of cellular responses to substance P(10, 11) . Endocytosis of the thrombin receptor also contributes to desensitization(12) . In contrast, endocytosis of the β_2-adrenergic receptor is not involved in rapid desensitization, but is required for resensitization(13, 14) . Thus, for several G-protein coupled receptors, endocytosis may regulate cellular responses to ligand.

Endocytosis of the GRP-R has usually been studied indirectly by ligand binding experiments using ^{125}I-labeled ligand and an acid wash procedure to distinguish between cell surface and internalized peptide(15, 16, 17, 18) . However, the intracellular compartment containing GRP and the GRP-R has not been identified, and the mechanism of endocytosis and sorting has not been examined. We directly observed endocytosis of both GRP and the GRP-R in transfected cells using fluorescent GRP and receptor antibodies. The results show that GRP and its receptor are rapidly internalized by a clathrin-dependent mechanism into early endosomes. GRP is sorted from its receptor in an acidified perinuclear compartment; GRP remains in the cell whereas the GRP-R recycles to the plasma membrane.

EXPERIMENTAL PROCEDURES

Reagents

Lysine extended human GRP-27 (Lys^0 GRP-27) was a gift from Joseph R. Reeve, UCLA. GRP-10 and GRP-27 were from Peninsula Laboratories Inc. (San Carlos, CA). ^{125}I-GRP-27 (2,000 Ci/mmol) was from Amersham Corp. Bis-functional cyanine 3.18 was a gift from Dr. Lauren Ernst (Biological Detection Systems, Pittsburgh, PA). FITC-labeled human transferrin, fura-2/AM, propidium iodide, and Slow Fade were from Molecular Probes (Eugene, OR). Kirsten murine sarcoma virus transformed rat kidney cells (KNRK) were from American Type Tissue Culture Collection (ATCC CRL 1569, Rockville, MD). Lipofectin reagent, G418, and cell dissociation buffer (phosphate-buffered saline (PBS) based, enzyme-free) were from Life Technologies, Inc./BRL (Gaithersburg, MD). Bafilomycin A_1 was a gift from Dr. Jonathan R. Green, Ciba-Geigy Ltd. (Basel, Switzerland). Other reagents were from Sigma.

Antibodies

The generation and characterization of an antiserum (#9342) to the C-terminal 12 amino acid residues of the mouse GRP-R (LINRNICHEGYV) conjugated to keyhole limpet hemocyanin has been described(19) . The antiserum was affinity-purified before use. A murine monoclonal antibody (M2) directed against the Flag peptide, was from International Biotechnologies, Inc. (New Haven, CT). Affinity-purified FITC- or rhodamine-conjugated goat anti-mouse IgG was from Cappel Research Products (Durham, NC). ^{125}I-Labeled sheep anti-mouse Ig (species specific F(ab`)$_2$) was from Amersham Corp.

Generation and Characterization of Cy3-GRP

Lys^0 GRP-27 (0.25 mM) was labeled with cyanine 3.18 and purified by reverse-phase high pressure liquid chromatography (HPLC), as described previously for cy3-substance P(11) . The absorbance of the eluent was monitored at 214 nm and fractions were collected for quantification by amino acid composition analysis. Mass spectrometry was used to determine the molecular weight (UCSF Mass Spectrometry Facility).

Expression Vectors and Cell Lines

KNRK cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/liter of glucose, containing 5 or 10% (v/v) fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were transfected with cDNA encoding an epitope-labeled chimera composed of the mouse GRP-R and an N-terminal Flag peptide (DYKDDDDK) in a neomycin-resistant expression vector (pcDNA Neo-1) (a gift from Dr. Thomas Segerson, Vollum Institute, Portland, OR), using Lipofectin as described(20, 21) . For comparison, KNRK cells were also transfected with the native rat GRP-R(19) . KNRK cells transfected with pRC/CMV served as a vector only control(21) . Positive clones were maintained in medium containing 400 µg/ml G418, and were characterized by immunofluorescence, ligand binding assays, and measurement of Ca^{2+} mobilization. The clones KNRK Flag GRP-R#25 and KNRK GRPR#4 expressed the highest level of the GRP-R and were used for all experiments. Most of the experiments used cells expressing the Flag receptor, but similar results were obtained with cells expressing the native rat receptor. Cells were plated 24-48 h before experiments on poly-L-lysine-coated glass coverslips (for microscopy or Ca^{2+} mobilization), on 16-mm plastic wells (for quantification of surface GRP-R), or on 30-mm plastic wells (for ^{125}I-GRP binding experiments). Unless stated otherwise, cells were incubated in DMEM containing 0.1% bovine serum albumin (DMEM/BSA) during all experiments.

Measurement of Ca^{2+} Mobilization

Cells were washed with Hank's buffered salt solution containing 0.1% bovine serum albumin, loaded with 2.5 µM fura-2/AM for 20 min at 37 °C, and washed(21) . Fluorescence was measured in a fluorimeter (F-2000, Hitachi Instruments, Irvine, CA) at 340 and 380 nm excitation and 510 nm emission. A single injection of GRP-10 or cy3-GRP (10 pM-1 µM) was followed by addition of digitonin (50 µM) to lyse cells, and then EGTA (5 mM).

Flow Cytometry

Cells were dissociated using an enzyme-free cell dissociation buffer, adjusted to 10^6 per tube in DMEM with 10% fetal calf serum, washed, and incubated with 100 nM cy3-GRP in 500 µl of DMEM with 10% fetal calf serum for 60 min at 4 °C. They were washed, resuspended in 200 µl of the dissociation buffer with 0.3% fetal calf serum and 1.5 µg/ml of propidium iodide, and analyzed using a FACS Star^{PLUS}

(Becton-Dickinson, Mountain View, CA), equipped with dual argon and helium-neon lasers. A minimum of 10,000 events were analyzed per sample. Viability of cells, as determined by exclusion of propidium iodide, exceeded 80%.

Microscopical Examination of Internalization of Cy3-GRP, FITC-transferrin, and the GRP-R

Cells were incubated with 100 nM cy3-GRP at 4 °C, to allow equilibrium binding, and were washed at 4 °C. Medium at 37 °C was added, and cells were incubated at 37 °C for 0-120 min to allow internalization. Cells were fixed with 4% paraformaldehyde in PBS for 20 min at 4 °C, and mounted in Slow Fade. To examine the specificity of cy3-GRP binding, cells were preincubated with 1 μM unlabeled GRP-10 for 30 min at 4 °C, and then incubated with 1 μM unlabeled GRP-10 and 100 nM cy3-GRP for 60 min at 4 °C. To examine endocytosis of cy3-GRP in living cells, cells were incubated with cy3-GRP as described, washed, and then incubated at 37 or 18 °C on an inverted microscope equipped with a microincubator (PDMI-2, Medical Systems Corp., Greenvale, NY). To simultaneously observe endocytosis of GRP and transferrin, cells were incubated with 100 nM cy3-GRP and 17 μM iron-loaded FITC-transferrin (22) at 4 °C, warmed to 37 °C for 0-10 min, and fixed as described.

To localize the internalized receptor, cells were incubated with 10-100 nM GRP-10 at 4 °C, washed, and incubated at 37 °C for 0-120 min. Cells were fixed with paraformaldehyde, and incubated in PBS containing 1% normal goat serum and 0.1% saponin for 3 periods of 5 min. All subsequent washes and antibody dilutions used this buffer. Cells were incubated with the GRP-R antiserum #9432 (1:50 for 4 h at 37 °C or 1:500 overnight at 4 °C), washed, incubated with a FITC- or rhodamine-labeled goat anti-rabbit IgG (1:50 for 2 h at room temperature), washed, and mounted.

Microscopy and Image Analysis

Fixed cells were examined using a Zeiss Axioplan microscope, equipped with fluorescein (Zeiss 487910, excitation 450-490 nm, emission 515-565 nm, chromatic beam splitter 510 nm) and rhodamine (Zeiss 487915, excitation 546 nm, emission>590 nm, chromatic beam splitter 580 nm) filters. Endocytosis in living cells was observed using a Zeiss Axiovert microscope equipped with a rhodamine filter. Images were either captured using a cooled CCD color video camera system (ZVS-47EC, Optronics Engineering, Goleta, CA) directly to a Macintosh Centris 650 computer using a ColorSnap 32+ video capture board (Computer Friends Inc., Portland, OR) (image size 640 × 480 pixels) or obtained using a Zeiss MC100 camera and Ektachrome EPH p1600 film, and transferred to Photo CD (Kodak). For confocal microscopy, we used a Zeiss Laser Scan Inverted 410 microscope with an argon-krypton laser with fluorescein (line selection 485/20 nm, main dichroic 510 nm, emission LP 515 nm) and rhodamine (line selection 530-585 nm, main dichroic 580 nm, emission LP 568 nm) filters, yielding images that were 512 × 512 pixels. All images were processed using Adobe Photoshop 2.5 (Adobe Systems Inc., Mountain View, CA) on a Macintosh Centris 650 computer, and photographed using an Image Corder slide maker (Focus Graphics, Foster City, CA).

Binding and Internalization of ^{125}I-GRP

Cells were washed with binding buffer (1:1 DMEM and Weymouth's medium MB 752/1 plus 8.2 g/liter BES, containing 0.1% BSA and 0.1% bacitracin, pH 7.4), and incubated with ^{125}I-GRP (400,000 cpm) and 0.039-10 nM unlabeled GRP-27 in 1 ml of binding buffer for 60 min at 4 °C, to allow binding to reach equilibrium(19) . Cells were washed with PBS containing 0.1% BSA, lysed in 2% Na_2HCO_3, 1% SDS, and 0.1 M NaOH for 10 min at 37 °C and counted. Specific counts were obtained by subtraction of nonspecific binding (cells pretreated with 1 μM GRP-27 prior to incubation with ^{125}I-GRP). Results were expressed as femtomole of ^{125}I-GRP bound per 10^6 cells and k_d and B_{max} were determined by Scatchard

analysis.

To quantify internalized peptide, cells were incubated with 400,000 cpm of ^{125}I-GRP and 2 nM unlabeled GRP-27 for 60 min at 4 °C, and washed 3 times at 4 °C. Warm medium was added, and the cells were incubated at 37 °C for 0-60 min, and washed 3 times with ice-cold buffer. To separate cell-surface (acid-sensitive) from internalized (acid-resistant) ^{125}I-GRP, cells were incubated in 1 ml of ice-cold 0.2 M acetic acid, 500 mM NaCl (pH 2.5) for 5 min, lysed, and the acid-sensitive and acid-resistant pools were counted(11, 19) .

Quantification of Cell-surface Flag Immunoreactivity

Cells were incubated with 0.01-1 μM GRP-10 in 250 μl of DMEM/BSA for 60 min at 4 °C, washed, and incubated at 37 °C for 0-60 min. They were then incubated with 0.1 μg/ml Flag antibody for 60 min at 4 °C, washed 3 times for 5 min at 4 °C, and incubated with 0.1 μCi/well of ^{125}I-sheep anti-mouse IG for 60 min at 4 °C. Cells were washed in PBS at 4 °C, and lysed with 0.5 M NaOH overnight at room temperature. Radioactivity and protein content of the lysate were measured. Specific binding was determined by subtracting nonspecific binding to KNRK CMV cells from total binding. To calculate the extent of GRP-induced internalization, we compared the specific binding of KNRK Flag GRP-R cells treated with GRP to that of cells incubated with medium alone, but otherwise treated identically.

Drug Treatments

Endocytosis can be inhibited by hyperosmolar sucrose, which blocks formation of clathrin-coated pits and interferes with bulk phase endocytosis in fibroblasts(23) , and by phenylarsine oxide, which cross-links sulfur groups(24) . The effects of these agents on endocytosis of GRP and GRP-R were examined. Cells were either preincubated with 0.45 M sucrose for 30 min at 37 °C and 0.45 M sucrose was added to all solutions, or preincubated with 80 μM phenylarsine oxide for 5 min only, washed, and incubated in DMEM/BSA for 30 min at 37 °C before the experiment(11) .

The effects of cycloheximide, brefeldin A, and various acidotropic agents on recovery of cell-surface GRP-R were examined. Brefeldin A causes disassembly of the Golgi apparatus and mixing with the endoplasmic reticulum, and induces alterations in the morphological appearance of endosomes and lysosomes(25) . Acidotropic agents used included bafilomycin A_1, an inhibitor of vacuolar-type H^+-ATPase(26) , and monensin, which prevents intracellular degradation of ^{125}I-substance P(11) . Cells were preincubated with 70 μM cycloheximide, 1 μM bafilomycin A_1, 50 μM monensin, or 10 μg/ml brefeldin A for 30-60 min at 37 °C before addition of GRP, and the drugs were included in all solutions during the experiment. Control cells were incubated with appropriate carrier solutions.

Statistical Analysis

Results are expressed as mean ± S.E. Differences between multiple groups are examined by an analysis of variance and a Student-Newman Keuls test or a Bonferroni *t* test. A $p < 0.05$ is considered statistically significant.

RESULTS AND DISCUSSION

The Flag GRP-R Was Fully Functional

The Flag GRP-R was fully functional, as assessed by binding experiments using ^{125}I-GRP and by measurement of GRP-induced Ca^{2+} mobilization. Scatchard analysis indicated that KNRK Flag GRP-R cells expressed 0.3×10^6 sites per cell with an apparent k_d of 0.7 nM. The EC_{50} for GRP-induced Ca^{2+} mobilization was 0.42 nM. KNRK GRP-R cells expressed 2×10^6 sites per cells with a similar k_d and EC $_{50}$ as the KNRK Flag GRP-R cells(19) . Thus, KNRK Flag GRP-R cells expressed high affinity GRP-Rs that were coupled to Ca^{2+} mobilization. We have previously shown that the Flag epitope does not affect ligand binding, Ca^{2+} mobilization, or endocytosis of the neurokinin 1 receptor(11, 20) .

Cy3-GRP Was Biologically Active and Specifically Interacted with the GRP-R

Lys^0 GRP-27 was readily labeled with cyanine 3.18 and the products were separated by HPLC (Fig. 1*A*). Unlabeled GRP eluted from the HPLC column after 18.1 min, and fluorescent products eluted at 16.6 min (peak 1), 19.1 min (peak 2), and 20.2 min (peak 3). Peak 2 was obtained with a higher yield and produced a stronger signal on binding, and was used in all further experiments. Amino acid composition analysis indicated that approximately 20% of the starting material eluted as peak 2, and confirmed that peak 2 was Lys^0 GRP-27. Mass spectrometry indicated a M_r of 3728.8, and that the peptide was labeled by a single cyanine molecule. The biological activity of GRP was not affected by the cyanine 3.18 group, since cy3-GRP induced a prompt increase in $[Ca^{2+}]_i$ in KNRK Flag GRP-R cells with an EC_{50} of 0.15 nM, similar to that of unlabeled GRP-10 (Fig. 1*B*). This result is not surprising, since only the C-terminal nonapeptide of GRP is required for full activity(27) , and both substance P and neurokinin A labeled with cyanine 3.18 retain full biological activity (11) . (2)

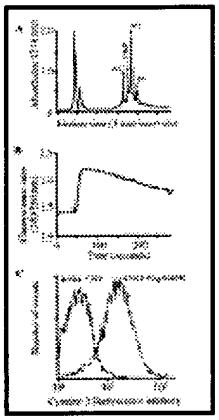

Figure 1: Purification and characterization of cy3-GRP. *A*, Lys^0 GRP-27 was labeled with cyanine 3.18, and purified by HPLC. Peak 2 was used in all experiments. *B*, peak 2 (1.75 nM) injected at the *arrow* induced a prompt increase in $[Ca^{2+}]_i$ in KNRK Flag GRP-R cells loaded with fura-2/AM. *C*, flow cytometry of cells after equilibrium binding of cy3-GRP indicated that the mean intensity of cyanine 3 in KNRK Flag GRP-R cells was 1 log greater than that of KNRK CMV cells.

The specificity of cy3-GRP binding to KNKR Flag GRP-R cells and the homogeneity of the cell population were examined by flow cytometry and fluorescence microscopy. KNRK Flag GRP-R cells incubated with cy3-GRP yielded at least 1 log fold greater intensity in the cyanine 3 channel than KNRK CMV control cells (Fig. 1*C*). At 4 °C, a strong, crisp signal was localized to the cell surface (Fig. 2*A*). No fluorescent signal was detected when KNRK Flag GRP-R cells were preincubated with 1 μM GRP-10 for 30 min at 4 °C, and then incubated with both cy3-GRP and 1 μM GRP-10 (Fig. 2*B*), or when KNRK-CMV cells were incubated with cy3-GRP. Since preincubation with excess unlabeled GRP blocked binding, cy3-GRP binds the GRP-R specifically in KNRK-Flag GRP-R cells.

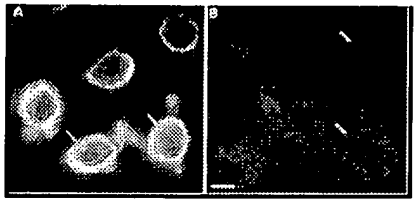

Figure 2: Specific binding of cy3-GRP to KNRK Flag GRP-R cells. *A*, cells were incubated with 100 nM cy3-GRP for 60 min at 4 °C, washed and fixed. *B*, cells were preincubated with 1 μM unlabeled GRP-10 for 30 min at 4 °C, and then incubated with 100 nM cy3-GRP plus 1 μM unlabeled GRP-10 as in *A*. *Scale bar* = 10 μm.

Biotinylated GRP-27 is also biologically active, and specifically interacts with the GRP-R on Swiss 3T3 cells(28) . However, use of this ligand requires a secondary detection system, which can cause artifacts in fluorescence microscopy and flow analysis, and necessitates extensive controls. Since cy3-GRP is directly visualized, it can be used for flow analysis and sorting of receptor bearing cells based directly on receptor affinity or number, and is also ideally suited for direct observation of peptide internalization in living cells.

Cy3-GRP and the GRP-R Were Rapidly Internalized at 37 °C

We directly observed endocytosis of cy3-GRP in living KNRK Flag GRP-R cells to examine the precise timing and pathway of internalization. Cells incubated with cy3-GRP at 4 °C were warmed to 37 °C, during which time endocytosis was continuously monitored and sequential images captured. At 4 °C, cy3-GRP was confined to the plasma membrane (Fig. 3*A*). Within 2 min at 37 °C, cy3-GRP was in numerous small vesicles underlying the cell surface, and there was diminished surface staining (Fig. 3*B*). After 10 and 30 min, cy3-GRP was evident in larger vesicles in a perinuclear region (Fig. 3, *C* and *D*). This process was uniform, since all cells in the field progressed at the same rate.

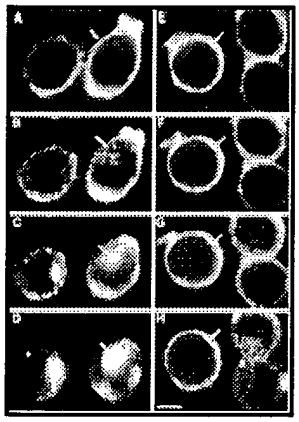

Figure 3: Internalization of the cy3-GRP by live KNRK Flag GRP-R cells. Cells were incubated with 100 nM cy3-GRP for 60 min at 4 °C, washed, and incubated on an inverted microscope at 37 °C (*A-D*) or 18 °C (*E-H*). Sequential images of the same cells (*A-D* or *E-H*) were captured after 0 (*A* and *E*), 2 (*B* and *F*), 5 (*C* and *G*), or 20 (*D* and *H*) min of warming. *Scale bar* = 5 μm.

In parallel experiments, after equilibrium binding of cy3-GRP (Fig. 3*E*), the temperature was raised to 18 °C, which is permissive for endocytosis but does not allow delivery to lysosomes (29) . Within 2 min at 18 °C, cy3-GRP was internalized into small vesicles beneath the cell surface (Fig. 3*F*). Cy3-GRP remained in small, peripherally located vesicles when the cells were maintained at 18 °C for up to 40 min (Fig. 3*H*, 20 min). When these same cells were warmed to 37 °C, cy3-GRP entered larger vesicles in a perinuclear region, suggesting that the larger more central vesicles are lysosomes. This agrees with previous studies showing that internalized ^{125}I-GRP is degraded in pancreatic acinar cells by a mechanism partially sensitive to the acidotropic agent chloroquine(17) .

Fluorescently-labeled transferrin, epidermal growth factor, and low density lipoprotein have been used to study endocytosis(22) . In contrast, small fluorescent peptides have not been widely used since they usually can be labeled on only 1 residue and therefore fluoresce weakly. Cy3-GRP not only generated a bright signal, but enabled sequential visualization of the same cell, which is often difficult with a rapidly photobleached fluorophore such as FITC. Based on our observations with cy3-GRP, we selected specific time points to examine endocytosis of GRP and its receptor in more detail.

We incubated KNRK Flag GRP-R cells with cy3-GRP and fixed the cells at various times after warming to 37 °C to (*a*) examine the uniformity of endocytosis in multiple cells; (*b*) investigate in detail the intracellular distribution of the peptide; and (*c*) make direct comparisons between these results and those showing the distribution of internalized GRP-R. In all cells examined, cy3-GRP was confined to the plasma membrane at 4 °C (Fig. 4*A*), and internalized into superficial vesicles after 5 min at 37 °C (Fig. 4*B*). After 10 min, cy3-GRP was predominantly localized in larger vesicles although also present in small superficial vesicles (Fig. 4*C*). By 30 min, cy3-GRP was found in large vesicles near the nucleus. At 60 and 120 min, cy3-GRP was observed in large, central vesicles and there was also diffuse central staining (Fig. 4*D*). Labeling of the plasma membrane was not observed at the later time points, suggesting that GRP did not recycle to the cell surface.

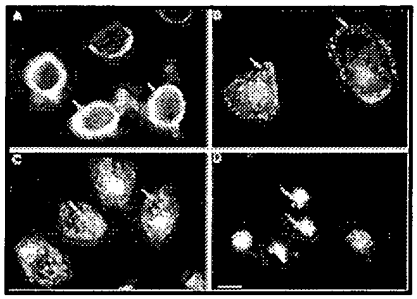

Figure 4: Internalization of the cy3-GRP by KNRK Flag GRP-R cells. Cells were incubated with 100 nM cy3-GRP for 60 min at 4 °C, washed, and incubated at 37 °C for 0 (*A*), 5 (*B*), 10 (*C*), or 60 (*D*) min. Cells were fixed and observed. *Scale bar* = 10 μm.

We compared the distribution of the GRP-R with that of cy3-GRP at the same times. KNRK Flag GRP-R cells were incubated with unlabeled GRP-10 at 4 °C, and warmed to 37 °C. At 4 °C, GRP-R immunoreactivity, like cy3-GRP, was confined to the plasma membrane (Fig. 5*A*). After 5 min at 37 °C, GRP-R was detected in numerous small vesicles located beneath the cell surface, similar to those containing cy3-GRP (Fig. 5*B*). After 10 min, the GRP-R was present in vesicles in a perinuclear region (Fig. 5*C*). After 30 and 60 min the intracellular staining had declined, and the plasma membrane was again strongly stained (Fig. 5*D*). This recovery of surface GRP-R immunoreactivity is in contrast to the distribution of cy3-GRP, which remained inside the cell.

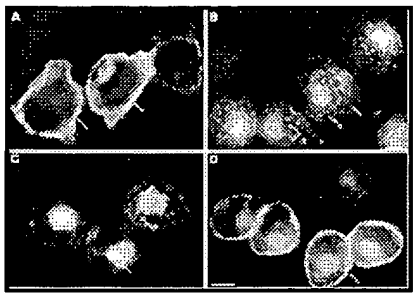

Figure 5: Internalization of the GRP-R in KNRK Flag GRP-R cells. Cells were incubated with 10 nM GRP-10 for 60 min at 4 °C, washed, and incubated at 37 °C for 0 (*A*), 5 (*B*), 10 (*C*), or 30 (*D*) min. Cells were fixed and incubated with the GRP-R antibody. *Scale bar* = 10 μm.

We used confocal microscopy to confirm that the punctate pattern of staining was due to internalization

of the receptor into vesicles rather than its aggregation in the plasma membrane, and to more precisely examine the distribution of the receptor. At 4 °C, GRP-R immunoreactivity was confined to the plasma membrane in optical sections just below the plasma membrane and through the center of the cell (Fig. 6*A*). No large pools of preformed intracellular GRP-R were detectable. After 5 min at 37 °C, the GRP-R was localized to numerous vesicles located immediately beneath the plasma membrane, and to small vesicles in the perinuclear region (Fig. 6*B*). After 10 min, the GRP-R was predominantly found in larger perinuclear vesicles (Fig. 6*C*). In contrast to cy3-GRP, which was completely depleted from the cell surface after 10 min at 37 °C (Fig. 4*C*), residual GRP-R remained at the plasma membrane. After 60 min, the GRP-R was localized to the plasma membrane and to small vesicles between the nucleus and plasma membrane (Fig. 6*D*).

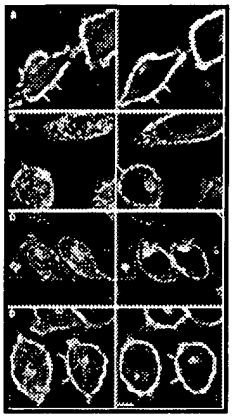

Figure 6: Confocal photomicrographs showing internalization of the GRP-R in KNRK GRPR cells. Cells were incubated with 10 nM GRP-10 for 60 min at 4 °C, washed, and incubated at 37 °C for 0 (*A*), 5 (*B*), 10 (*C*), or 60 (*D*) min. Cells were fixed and incubated with the GRP-R antibody. The figure shows optical sections at ~3.5-μm intervals through the base (*left panels*) and center (*right panels*) of the cells. *Scale bar* = 10 μm.

Thus, the primary steady-state location of the GRP-R in KNRK cells is the plasma membrane. Incubation with GRP and warming to 37 °C alters the steady state distribution by causing internalization. Initially, cy3-GRP and the GRP-R are internalized into superficial vesicles which rapidly progress to a perinuclear region where cy3-GRP and the GRP-R are sorted into distinct compartments. After 60 min the GRP-R resumes the steady state distribution, whereas cy3-GRP remains inside the cell. We have previously shown that substance P and the neurokinin 1 receptor are internalized into the same endosomes and then sorted in a perinuclear region, leading to degradation of substance P and recycling of the neurokinin 1 receptor(10, 11) . ([3])Therefore, it is probable that GRP is also degraded in a perinuclear region and its receptor returns to the cell surface.

GRP and Transferrin Were Internalized into the Same Endosomes

Transferrin and the transferrin receptor are constitutively internalized into early endosomes and rapidly recycle together to the cell surface(30, 31) . To determine whether GRP was internalized into endosomes containing a recycling receptor, we incubated KNRK Flag GRP-R cells with cy3-GRP and FITC-transferrin at 4 °C, warmed them to 37 °C, and examined them by confocal microscopy. At 4 °C, both cy3-GRP and FITC-transferrin were confined to the cell surface. However, after 5-10 min incubation at 37 °C, there was a reduction in the intensity of cell-surface staining for cy3-GRP and FITC-transferrin, and both fluorophores were localized in numerous vesicles beneath the plasma membrane (Fig. 7, *A* and *B*). Colocalization was confirmed by superimposing images of the same field for cy3-GRP and the FITC-transferrin, yielding a yellow image (Fig. 7*C*). This colocalization shows that GRP and its receptor were first present in early endosomes(30, 31) . We have previously shown that substance P and the neurokinin 1 receptor are also internalized into endosomes containing transferrin receptor(11) . Therefore, internalization into endosomes containing recycling receptors may be a common theme for neuropeptide receptors.

Figure 7: Confocal photomicrographs showing internalization of cy3-GRP and FITC-transferrin by KNRK Flag GRP-R cells. Cells were incubated with 100 nM cy3-GRP and 17 μM FITC-transferrin for 60 min at 4 °C, washed, and then incubated for 10 min at 37 °C. Cells were fixed and observed using fluorescein filters to detect FITC-transferrin (*A*) and rhodamine filters to detect cy3-GRP (*B*). The image in *C* is the superimposition of images from *A* and *B*. Colocalization of FITC-transferrin and cy3-GRP is indicated by *arrows* pointing to the yellow vesicles. *Scale bar* = 10 μm.

Quantification of Internalization of ^{125}I-GRP and the GRP-R

We examined the rate and extent of GRP internalization in binding experiments using ^{125}I-GRP. When KNRK Flag GRP-R cells were incubated with ^{125}I-GRP at 4 °C, 116.5 ± 1.2 fmol of GRP were specifically bound per 10^6 cells (duplicate observations, n = three experiments). At this time, 109.3 ± 0.9 fmol were in the acid-sensitive (cell surface) fraction and 7.2 ± 0.3 fmol were in the acid-resistant (internalized) fraction. Rapid internalization of ^{125}I-GRP occurred when the temperature was raised to 37 °C (Fig. 8*A*). After 20 min, when internalization was maximal, 62.3 ± 1.3 fmol of GRP were in the acid-sensitive fraction and 168.0 ± 1.0 fmol were in the acid-resistant fraction. At 30 min, the amount of GRP at the cell surface continued to decline. Thus, ^{125}I-GRP, like cy3-GRP, is rapidly internalized and does not return in any great proportion to the cell surface. Internalization of ^{125}I-GRP is dependent on serines and threonines in the GRP-R, although the tyrosine in the highly conserved NPX_nY motif is not required(19, 32) .

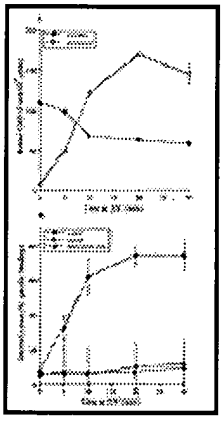

Figure 8: Internalization of ^{125}I-GRP by KNRK Flag GRP-R cells. *A*, cells were incubated with ^{125}I-GRP for 60 min at 4 °C, washed, and incubated at 37 °C. An acid wash was used to separate acid-sensitive (cell surface) and acid-resistant (internalized) fractions. *B*, the effects of 0.45 M sucrose and 80 μM phenylarsine oxide on internalization of ^{125}I-GRP in KNRK Flag GRP-R cells. Binding is expressed as a percentage of the counts added to the cells, normalized to a cell count of 100,000. Results are of duplicate observations from n = three experiments.

To quantify cell surface GRP-R, we incubated non-permeabilized KNRK Flag GRP-R cells with an antibody to the extracellular Flag epitope followed by a ^{125}I-labeled secondary antibody. Nonspecific binding was measured by incubating KNRK CMV cells with the Flag antibody, and was 6.8 ± 1.0% of that measured in KNRK Flag GRP-R cells (triplicate observations, n = 9 experiments). Specific binding of the Flag antibody to KNRK Flag GRP-R cells that were incubated with or without GRP-10 for 60 min at 4 °C was the same. Therefore, GRP binding did not affect the ability of the Flag antibody to bind the receptor. When KNRK Flag GRP-R cells were incubated with 100 nM GRP-10 at 4 °C, and warmed to 37 °C for various times, there was a marked reduction in cell surface Flag immunoreactivity which then returned to baseline levels (Fig. 9*A*). After 10 min at 37 °C, the surface immunoreactivity had declined to

77.3 ± 2.7% (triplicate observations, n = 13 experiments) of control levels (no GRP-10), and after 60 min at 37 °C had returned to 92.08 ± 1.86% (n = 10 experiments) of the control. The loss of surface Flag immunoreactivity at 10 min was dependent on the concentration of ligand and was maximal at 1 μM GRP-10. The recovery rate of surface Flag immunoreactivity was not altered by ligand concentration.

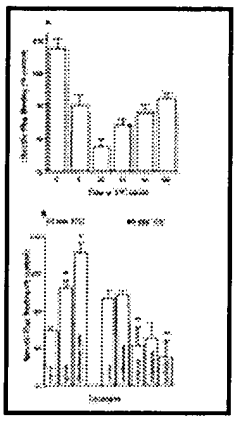

Figure 9: Quantification of surface Flag immunoreactivity in KNRK Flag GRP-R cells. *A*, cells were incubated with 100 nM GRP-10 for 60 min at 4 °C, washed, warmed to 37 °C for 0-60 min, and incubated at 4 °C with the Flag antibody and ^{125}I-sheep anti-mouse IG. At each time point, specific Flag binding is expressed as a percentage of binding to control cells not treated with GRP. Results are of triplicate observations from n = 3-13 experiments. *B*, to study the mechanism of endocytosis, cells were treated with 0.45 M sucrose or 80 μM phenylarsine oxide, and after equilibrium binding of 100 nM GRP-10 at 4 °C, were warmed to 37 °C for 10 min. To study the return in surface GRP-R, cells were treated 70 μM cycloheximide, 50 μM monensin, 1 μM bafilomycin A_1, or 10 μg/ml brefeldin A, and after GRP binding, warmed to 37 °C for 60 min. Surface GRP-R were detected as described in *A*. Each experimental group had its own control not exposed to GRP, to correct for any effects of these agents on the assay. Results are from triplicate observations from n = three to nine experiments. *, <0.05 compared to the 10-min carrier. **, <0.05 compared to the 60-min carrier.

Together, these results show that GRP and the GRP-R are maximally internalized within 10-20 min at 37 °C. However, whereas most of the specifically bound ligand is internalized, a considerable amount of receptor remains at the cell surface even after exposure to high concentrations of GRP (1 μM). Residual surface receptors may be in a low affinity state and unable to bind ligand.

Hyperosmolar Sucrose and Phenylarsine Oxide Inhibited Internalization of ^{125}I-GRP and the GRP-R

Treatment of cells with hyperosmolar sucrose or with phenylarsine oxide inhibited internalization of ^{125}I-GRP (Fig. 8*B*). After 10 min incubation at 37 °C, only 10.4 ± 5.6% of bound GRP was in the acid-resistant fraction of cells treated with hyperosmolar sucrose, and 5.6 ± 6.8% of bound GRP was in the acid-resistant fraction of cells treated with phenylarsine oxide. In untreated cells, 61.6 ± 10.4% of bound GRP was present in the acid-resistant fraction at this time. In cells treated with hyperosmolar sucrose or phenylarsine oxide, the acid-resistant fraction remained low throughout the experiment.

Similarly, treating cells with hyperosmolar sucrose or phenylarsine oxide inhibited the decline in cell surface Flag immunoreactivity. When carrier-treated KNRK Flag GRP-R cells were incubated with 100 nM GRP-10 for at 4 °C, washed, and warmed to 37 °C for 10 min, surface Flag immunoreactivity had declined to 84.7 ± 1.0% (triplicate observations, n = four experiments) of control levels (no GRP-10). In cells treated with 0.45 M sucrose or 80 μM phenylarsine oxide, surface Flag immunoreactivity was 96.1 ± 2.0 and 100.5 ± 2.7% of control levels, respectively. In parallel experiments, KNRK Flag GRP-R cells treated with sucrose or phenylarsine oxide were processed to localize the GRP-R by immunocytochemistry and examined by confocal microscopy. Internalization of GRP-R immunoreactivity into early endosomes was abolished by both agents (Fig. 10).

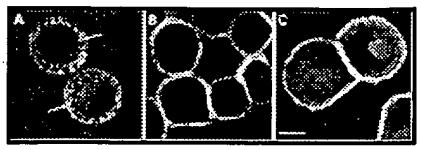

Figure 10: Confocal photomicrographs showing the effects of hyperosmolar sucrose and phenylarsine oxide on internalization of the GRP-R in KNRK GRPR cells. Cells were incubated with 10 nM GRP-10 for 60 min at 4 °C, washed, and incubated at 37 °C for 10 min. Cells were immediately fixed and incubated with the GRP-R antibody. *A,* control cells; *B,* cells treated with 0.45 M sucrose; *C,* cells treated with 80 μM phenylarsine oxide. *Scale bar* = 5 μm.

Therefore, GRP and its receptor are internalized by clathrin-coated pits. This is a common theme for seven transmembrane domain receptors, since ligand-induced endocytosis of the neurokinin 1, β_2-adrenergic, and thrombin receptors is mediated by clathrin(11, 12, 13) . A clathrin-independent mode of internalization exists for glycosylphosphatidylinositol-anchored proteins, such as the folate receptor(33) . The folate receptor clusters in caveolae with underlying caveolin enabling bound folate to enter the cytoplasm. Ligands internalized through both clathrin-dependent and clathrin-independent mechanisms move into endosomes containing the transferrin receptor and follow a common pathway(34, 35) .

Internalized GRP-R Recycled to the Cell Surface

The recovery of GRP-R immunoreactivity at the plasma membrane that was observed both by immunofluorescence and in the Flag binding experiments may represent recycling of internalized receptors to the cell surface, or the migration of preformed or newly synthesized receptors to the plasma membrane. Immunofluorescence experiments did not reveal large intracellular reserves of the GRP-R, suggesting that the migration of preformed receptors to the plasma membrane did not account for this recovery. To discriminate between new receptor synthesis and recycling of internalized receptor, cells were treated with cycloheximide, to inhibit protein synthesis, or acidotropic agents, to block sorting and possible recycling of the receptor, and surface Flag immunoreactivity was determined. In control experiments on carrier-treated cells, Flag immunoreactivity at 60 min was 93.8 ± 2.0% (triplicate observations, n = nine experiments) of that observed at the same time in cells that were not treated with GRP-10 (Fig. 9*B*). This recovery was unaffected by cycloheximide, and is thus independent of new protein synthesis (Fig. 9*B*). These results agree with previous studies, where resensitization of the GRP-R in dispersed pancreatic acini was not sensitive to cycloheximide(7) . However, both bafilomycin A_1 and monensin attenuated the return of surface Flag immunoreactivity (Fig. 9*B*). These experiments suggest that endosomal acidification is required for sorting of the GRP-R into a recycling pathway. We did not examine the pH of early endosomes or sorting compartments, but others have shown that acidification below pH 6.5 reduces binding and accelerates dissociation of ^{125}I-bombesin from the GRP receptor(8, 16) . Brefeldin A also attenuated the recovery of cell surface Flag immunoreactivity (Fig. 9*B*). Although brefeldin A disrupts the Golgi apparatus, it also induces alterations in the morphological appearance of endosomes and lysosomes(25) . Therefore, the inhibition of recovery may have been caused by several effects of brefeldin A.

Physiological Relevance of Endocytosis of the GRP-R

Endocytosis of the GRP-R may contribute to desensitization by depleting the plasma membrane of high affinity receptors. Indeed, internalization of the neurokinin 1 and thrombin receptors is sufficiently rapid to account for desensitization(10, 12) . However, desensitization of the β_2-adrenergic receptor is

mediated by phosphorylation by β-adrenergic receptor kinase and protein kinase A which uncouples the receptor from G proteins(36, 37) . Indeed, mutant β_2-adrenergic receptors that do not internalize still desensitize(14) . We do not know whether the GRP-R is also a substrate for a G-protein receptor kinase and the role of phosphorylation in desensitization of the NK1-R is unknown. Resensitization of the β_2-adrenergic receptor requires internalization and dephosphorylation(13) , and the recycling of the GRP-R to the plasma membrane may contribute to resensitization of cellular responses to GRP. Moreover, another growth factor, epidermal growth factor, induces DNA synthesis only if it is internalized, although it can induce RNA synthesis when at the cell surface(38) . Thus, internalization of the GRP-R may be important in mediating some of the biological effects of GRP.

We conclude that GRP induces clathrin-dependent internalization of the GRP-R into early endosomes. GRP and its receptor are sorted in an acidified, perinuclear compartment. GRP remains inside the cell, where it is probably degraded, whereas the receptor recycles to the plasma membrane. Internalization and recycling of the GRP-R may modulate the cellular response to GRP.

FOOTNOTES

* This work was supported by National Institutes of Health Grants DK 39957 (to N. W. B. and D. G. P.), DK 43207 (to N. W. B.), NS 21710 (to N. W. B. and D. G. P.), DK 42341 (to J. H. W.), and DK 35740 (to J. H. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore by hereby marked ``*advertisement*'' in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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(1) The abbreviations used are: GRP, gastrin releasing peptide; GRP-R, gastrin releasing peptide receptor; cy3-GRP, cyanine 3.18-labeled gastrin releasing peptide; KNRK cells, Kirsten murine sarcoma virus transformed rat kidney cells; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography; FITC, fluorescein isothiocyanate; BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; CMV, cytometalovirus.

(2) E. F. Grady and N. W. Bunnett, unpublished observations.

(3) E. F. Grady, A. M. Garland, P. D. Gamp, M. Lovett, D. G. Payan, and N. W. Bunnett, manuscript submitted.

ACKNOWLEDGEMENTS

We thank Dr. D. M. McDonald for use of the confocal microscope in the Core Microscopy Laboratory of

the Cardiovascular Research Institute, UCSF.

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